Biomarkers for Alzheimer disease – current status and prospects for the future

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Abstract

Accumulating data from clinical research support that the core Alzheimer disease (AD) cerebrospinal fluid (CSF) biomarkers amyloid β (Aβ42), total-tau (T-tau), and phosphorylated tau (P-tau) reflect key elements of AD pathophysiology. Numerous clinical studies support that these biomarkers contribute with diagnostically relevant information, also in the early disease stages, which has led to the CSF biomarkers having a central position in novel diagnostic criteria for the disease. Technical developments have made it possible to measure these biomarkers using fully automated assays with high precision and stability. Standardization efforts have given Certified Reference Materials for CSF Aβ42, with the aim to harmonize results between assay formats that would allow for uniform global reference limits and cut-off values. This progress will serve as the basis for a more general introduction of these diagnostic tests in clinical routine practice. However, the heterogeneity of pathology in late-onset AD calls for an expansion of the AD CSF biomarker toolbox with additional biomarkers reflecting additional aspects of AD pathophysiology. One promising candidate is the synaptic protein neurogranin that seems specific for AD and predicts future rate of cognitive deterioration. Further, blood biomarkers would be highly valuable as screening tools in the early diagnostic evaluation of patients with cognitive problems and suspected AD. In this respect, technical developments with ultrasensitive immunoassays and novel mass spectrometry techniques give promise of biomarkers to monitor brain amyloidosis (the Aβ42/40, or APP669-711/Aβ42 ratios), and neurodegeneration (tau and neurofilament light proteins) in plasma samples, but future studies are warranted to validate these promising results further.

This review paper gives an update on the research and development of cerebrospinal fluid (CSF) biomarkers for Alzheimer disease (AD) with focus on diagnostic applications. It is more than two decades since the most commonly used ELISA methods, the INNOTEST assays, for quantification of total tau (T-tau), phosphorylated tau (P-tau) and amyloid β (Aβ42) in CSF were published (1-3), showing increased levels of T-tau and P-tau together with decreased Aβ42, a biomarker pattern often called the "Alzheimer CSF profile". These core AD CSF biomarkers have made a journey from the first studies based on research grade assays to their current status with assays on fully automated instruments and extensive clinical validation of diagnostic performance. Recent developments have also given some novel candidate biomarkers for synaptic degeneration, another key aspect of AD pathophysiology. Last, technical developments of novel ultrasensitive immunoassay and mass spectrometry methods show promise for blood biomarkers with potential applications as screening tools for neurodegeneration and brain amyloidosis.

Molecular neuropathology of Alzheimer's disease

Ever since the first case report on AD, plaques and tangles, the neuropathological hallmarks of AD, were known to be composed of "amyloid", i.e. proteinaceous deposits that can be stained with dyes such as Congo Red. However, efforts to identify their protein composition were for a long time unsuccessful, largely due to the lack of methods to purify plaques and tangles and the insolubility of the amyloid fibrils. After the development of a method to purify amyloid plaques (4), a paper in 1985 published the full sequence of a 40 amino acid (4 kDa) protein purified from plaques in AD brain tissue (5). Based on its molecular weight, the protein was called amyloid A4 protein but today we know it as β-amyloid or Aβ. The identification of Aβ (specifically its amino acid sequence) facilitated the cloning of the amyloid precursor protein (*APP*) gene (6). Importantly, the Aβ domain of 42-43 amino acids within the APP protein (a transmembrane protein with a single transmembrane domain) was predicted to be partially embedded in the plasma membrane (6), and thus that cleavage of APP by two putative enzymes, called β-secretase (today identified as BACE1) and γ-secretase (today identified as the presenilin complex) was needed to generate Aβ (7).

Tau protein is microtubule-associated protein primarily located in the neuronal axons, which due to alternative splicing has 6 isoforms, with 352 to 441 amino acids, and with molecular weights of 50 to 65 kDa (8). In 1986, it was shown that tangles are composed of abnormally hyperphosphorylated and truncated tau protein (9) with around three times more phosphorylated sites than normal tau. The hyperphosphorylation is believed to abrogate the normal function of tau to bind to and stabilize the microtubules in the neurons (10), leading to

disruption of the microtubules and impairment of axoplasmic flow and loss of neuronal connectivity. Except for neurofibrillary tangles, aggregated hyperphosphorylated tau is also found in neuropil threads and in the dystrophic neurites surrounding amyloid plaques.

Pathophysiological rationale for Alzheimer's disease fluid biomarkers

After the first case description of the disease in 1906, AD was regarded as a "presenile" dementia, affecting people between 50-65 years of age, while older individuals with dementia were believed to have "senile dementia", *i.e.*, cognitive deterioration as a part of more or less normal ageing. Around 1970, a number of publications showed that people with "senile dementia" exhibited the same type of pathology (amyloid plaques and tangles) as those with presenile AD (11, 12). After this, senile dementia in older patients was called senile dementia of the Alzheimer type (SDAT) or late onset AD (LOAD), while patients with onset before 65 years of age were diagnosed with early onset AD (EOAD). However, with time these borders bleached out, and all cases was termed AD, irrespective of age, for review see (13).

However, several studies have shown that the severity of AD (*i.e.*, plaque and tangle) pathology is greatest in early onset AD patients, while in late onset cases the severity of neuropathological changes varies considerably between patients, and in higher ages the level of changes overlaps with that found in cognitively unimpaired elderly (14-18). Further, after the discoveries of other types of age-related proteinopathies than tau pathology and Aβ plaques, studies have shown that most late onset clinically diagnosed AD cases do not have pure AD (plaque and tangle) pathology, but also varying severities and combinations of α-synculein and TDP-43 deposits and additional microvascular changes and hippocampal sclerosis (19, 20). This pathophysiological heterogeneity also makes clinical symptomatology of late onset AD variable and unspecific (13), and introduces diagnostic difficulties. Indeed, several papers have shown that also in cases that have undergone evaluations at expert research centres and followed clinically for years, the purely clinical diagnostic criteria for AD have poor accuracy, with sensitivity and specificity figures of around 70-80% when related to neuropathology (21, 22).

For these reasons, there is a large need of diagnostic tools to support the clinical diagnosis of AD. A correct clinical diagnosis of AD is currently important to initiate treatment with symptomatic drugs, including acetylcholine esterase (AChE) inhibitors and NMDA-receptor antagonists (23), and will be even more necessary the day disease-modifying drugs, such as secretase inhibitors or Aβ immunotherapies, hopefully will be available. In clinical medicine, fluid laboratory medicine biomarkers have a central position and influence up to 70% of clinical medical decisions (24). For

brain disorders, including AD, development of fluid biomarkers was initiated using CSF as the matrix, which compared with blood has the advantage of its proximity to the brain parenchyma, with brain proteins being secreted from the brain extracellular space to the CSF, which is accessible for CSF collection by lumbar puncture, for details see (25). However, for possible applications as screening tools in primary care, or for longitudinal evaluations with repeated sampling, blood biomarkers would be preferable, since blood is more accessible than CSF. Last, considering that elderly AD patients have multiple pathologies, a broader panel of fluid biomarkers reflecting not only amyloid and tau pathology would be needed.

CSF amyloid β as an Alzheimer biomarker

In 1992, it was shown that Aβ is secreted to the CSF (26), a finding that set the stage for developing quantitative immunoassays for Aβ in CSF. However, initial papers on "total" Aβ in CSF were disappointing, showing no or minor differences between AD patients and controls (27). Guided by the finding that Aβ deposited in both diffuse and core amyloid plaques extends to position 42 (Aβ42), which also is the Aβ species that is earliest deposited in plaques (28), the first ELISA method for Aβ42 was published in 1995, showing a marked reduction in CSF samples from AD patients (29). The decrease in CSF Aβ42 in AD dementia has been validated in numerous subsequent papers; a meta-analysis showed very consistent findings across 131 studies with a mean fold change of 0.56 for CSF Aβ42 compared with cognitively unimpaired elderly (30).

The basis for the decrease in CSF Aβ42 was unresolved for long, but in 2003, an autopsy study found an association between low post-mortem ventricular CSF Aβ42 levels and high plaque counts (31), a finding validated in a study revealing a correlation between reduced CSF Aβ42 measured in ante-mortem lumbar CSF samples and amyloid plaque counts measured autopsy (32). These results are hampered by analysis of post-mortem CSF samples or by the latency between ante-mortem CSF samples and autopsy measures at autopsy. However, the introduction of positron emission tomography (PET) ligands binding to fibrillar Aβ in the brain enabled studies on the relation between these amyloid biomarkers. In 2006, a paper presented the findings that elderly people, regardless of whether they had clinical AD or were cognitively unimpaired, who had low CSF Aβ42 levels also had positive amyloid PET scans, and vice versa (33). This matches the hypothesis that the pathophysiological basis for the reduction of CSF Aβ42 in AD is that this hydrophobic peptide aggregates and becomes sequestered in plaques, with lower amounts remaining to be secreted to the extracellular space and the CSF, resulting in lower CSF levels of Aβ42 (1). Indeed, even if hampered by uncertainties, the difference in CSF Aβ42 concentrations between AD patients and controls (in pg/mL) matches the amount of Aβ deposited in plaques in

the AD brain when considering the extent and weight of affected tissue, a duration (preclinical and clinical) of AD of around 30 years and the average CSF production per day and year (34).

In agreement, several papers have consistently found a high concordance between CSF Aβ42 and amyloid PET status (35). Except for cross-sectional studies, a high concordance between CSF Aβ42 and amyloid PET status was also shown in a large prospective and longitudinal clinical study on consecutive memory clinic patients (36). The figure for the concordance between CSF Aβ42 and amyloid PET status is around 90% in most studies (35), independent of which assay is used for measurement of CSF Aβ42 and whether comparisons are made with mean cortical SUVr or visual read of PET scans (35, 37). This figure is in the same range as the concordance between SUVr and visual read of PET scans, and between different readers of PET scans to dichotomize into positive and negative scans (37). A difference between CSF and PET biomarkers for brain amyloid deposition is that while the CSF Aβ42 concentration is given as digits, amyloid PET scans show the regional distribution of ligand retention. However, a clinical study comparing the diagnostic accuracy of CSF biomarkers and amyloid PET for diagnosing early AD showed no benefit of regional PET measures as compared with global neocortical ligand retention, which both were highly concordant with CSF Aβ42 (38). Discordancy in the form of low CSF Aβ42 but negative amyloid PET is mainly found in cognitively unimpaired elderly and early AD patients, while it is unusual in AD dementia cases (39). One study showed that non-demented individuals with low CSF Aβ42 but negative amyloid PET scans showed increased brain amyloid accumulation at a follow-up PET scan, similar to those who had positive amyloid PET at baseline, and at a rate three times higher than those with negative scans and normal CSF Aβ42 levels (40), suggesting that CSF Aβ42 may be an earlier biomarker for brain amyloidosis than amyloid PET. To sum up, the current literature suggests that CSF Aβ42 and amyloid PET can be used interchangeably in the clinic, the choice may be based on availability, costs, risk estimations (radiation exposure vs. post lumbar puncture headache), together with both physician and patient preferences.

Except for Aβ42, several other Aβ species are present in human CSF, with Aβ1-40 being the most abundant, found at around 10 times higher concentrations than Aβ42 (41, 42). In 1998, a first study showed that a combined analysis of Aβ42 and Aβ40 improved the diagnostic accuracy for AD (43). After this, numerous studies have shown that while CSF Aβ40 shows no, or minor, change in AD (30), the CSF Aβ42/Aβ40 ratio has higher performance to identify AD than CSF Aβ42 as a single biomarker (44-46). Recent studies also indicate that the Aβ42/Aβ40 ratio shows better concordance with amyloid PET positivity (47-49), and that the CSF Aβ42/Aβ40 ratio has diagnostic value in the clinical setting (50). The reason for the improved performance of the Aβ42/Aβ40 ratio is unclear, but it has been hypothesized that CSF Aβ40 can serve as a proxy for

"total" Aβ levels, and that the ratio normalizes for "total" Aβ production level between individuals, meaning that a reduction in CSF Aβ42 in individuals with high total Aβ production can be identified more accurately, and marginally low CSF Aβ42 in individuals with low total Aβ production will not be misinterpreted as indicative of brain amyloidosis (51). However, alternative explanations may be that the ratio normalizes for differences in CSF dynamics (having similar effects on Aβ42 and Aβ40), or for pre-analytical confounders affecting both Aβ42 and Aβ40.

CSF tau proteins as Alzheimer biomarkers

The finding that phosphorylated tau is the key component of tangles (9) made tau proteins in CSF candidate biomarkers for AD. Indeed, using the monoclonal antibody Alz-50, that reacts with PHF-tau and normal tau protein (52), in 1987 (Figure 1), tau protein was identified in AD CSF samples using Western blot (53), while levels were below detection limit in control CSF samples. This called for quantitative immunoassays to measure tau in CSF.

Total tau

In 1993, the first ELISA method for quantification of tau in CSF was published (54). This ELISA was based on the combination of a monoclonal anti-tau antibody against the mid-domain combined with a polyclonal anti-tau antiserum in the sandwich format (54). Two years later, the first sandwich ELISA method based only on monoclonal antibodies, known as the "Innogenetics" or "INNOTEST" assay, was published (2). This assay is based on mid-region monoclonal antibodies recognizing all six tau isoforms irrespective of phosphorylation state, and therefore got the label as a "total" tau (T-tau) assay (2). A marked increase in CSF t-tau was found in AD dementia patients, a finding that since then has been replicated in hundreds of papers, also using several other assay formats (30).

CSF T-tau has been proposed as a "state marker", reflecting the intensity of neurodegeneration or severity of acute neuronal damage (55). Indeed, following acute brain damage, CSF T-tau levels are dynamic and increase within days following injury and then stay elevated for weeks until normalization (56, 57), and in chronic neurodegenerative disorders, highest CSF T-tau levels are found in disorders with the most intense neurodegeneration, especially in Creutzfeldt-Jakob disease, where levels are 10-20 fold higher than in AD (58, 59). In the AD spectrum, higher CSF T-tau and P-tau predicts a more rapid clinical disease progression (37, 60-62), supporting CSF T-tau as a biomarker for intensity of neurodegeneration.

Phosphorylated tau

Measurement of tau protein in CSF that is phosphorylated at residues that is known to have this post-translational modification at the same residues in AD brain tissue may reflect tau pathology. The first assay for CSF P-tau ELISA method for quantification of tau phosphorylated at threonine 181+231 showed a marked increase in AD (2). A study using a modified assay, specific for tau phosphorylated at threonine 181 (P-tau181) confirmed this finding (3). In addition, a marked increase in CSF P-tau in AD has been shown for several other mid-domain phosphorylated tau residues, including threonine 231 and serine 235 as well as serine 199 (63), threonine 231 (64), and also for the C-terminal residues serine 396 and 404 (65). Studies specifically comparing different CSF P-tau assays as biomarkers for AD are few, but one report showed that CSF levels of P-Tau181, P-Tau199 and P-Tau231 correlate tightly, and had a similar performance to discriminate AD from other neurodegenerative disorders and non-demented controls (66).

Some studies have examined the relationship between CSF tau levels and neuropathology measures of tau pathology. Some studies report correlations between CSF levels of P-tau and neocortical tangle counts (67), but such associations may depend on the patient cohort examined, and will rely on whether cases with both low (stage 1-2) and high Braak stages are included in the correlations (32). In addition, several studies also show correlations between neuritic plaque counts and higher CSF T-tau (32) and P-tau (67) levels. The findings that CSF P-tau levels do not change with acute brain damage such as acute ischemic stroke (56) and is normal or only marginally increased (while CSF T-tau shows a massive increase) in neurodegenerative disorders with marked neurodegeneration but no tangles, such as Creutzfeldt-Jakob disease (58, 59), support that the CSF level of P-tau probably reflects the phosphorylation state of tau, and not simply neuronal damage or degeneration. Indeed, at the group level, high CSF P-tau is only found in AD and not in other neurodegenerative disorders.

Recently, PET has been developed to visualize tau pathology directly in patients. In 2016, two studies examined the relationships between tau proteins measured in CSF and tau deposits evaluated by PET scans, demonstrating that CSF and PET tau biomarkers show weak global correlations (68, 69). One study on cognitively normal elderly found that total cortical tau ligand binding correlates modestly with CSF P-tau, but not with T-tau (68), while the correlations were stronger with tau PET SUVRs in medial temporal lobe structures, the areas known to be affected earliest in AD. The correlation coefficients between the CSF biomarker and global tau PET metrics are stronger when including both controls and AD patients (70), apparently due to the large difference in both CSF tau levels and tau ligand retention between controls and AD patients. A recent study examining the relations between CSF tau levels, MRI measures of atrophy and tau PET showed that while tau PET correlated with degree of atrophy on MRI and severity of cognitive

impairment, CSF T-tau and P-tau were highly correlated, with high levels found in preclinical AD, despite normal tau PET scans (71). These findings support the concept that CSF T-tau and P-tau mainly are biomarkers of "disease state", neurodegeneration and tau phosphorylation state, respectively, and are increased also in earlier disease stages, before tau aggregates can be identified on PET scans. In contrast, tau PET is a biomarker of "disease stage", correlating with stage of brain atrophy and severity of cognitive deficits. Importantly, in a study enrolling also non-AD neurodegenerative disorders (progressive supranuclear palsy, non-fluent primary progressive aphasia, corticobasal syndrome and frontotemporal dementia), CSF tau proteins and tau PET both showed high differential diagnostic value (70). This suggests that it may be important to develop specific biomarkers for non-AD tau pathology.

CSF biomarkers for diagnosis of early Alzheimer's disease

It is logical to assume that disease-modifying drug candidates have a larger chance to show effectiveness if treatment can be initiated before neurodegeneration is too severe, *i.e.* in early the earliest symptomatic stages of the disease (72). In 1999, a first paper showed that MCI patients who a clinical follow-up investigations had deteriorated and developed AD dementia had high CSF levels of T-tau and low Aβ42 at the baseline investigation (73), but this paper did not include any group with MCI patients who did not deteriorate clinically. An extended clinical follow-up period is important to ascertain that so called "stable" MCI patients will not show cognitive worsening or develop dementia. In 2006, an article presented data on the core AD CSF biomarkers on a large prospective cohort of MCI patients with an extended (4-7 years) clinical follow-up period (74). This study showed a very high (95%) diagnostic sensitivity for the combination of low Aβ42 and high CSF T-tau/P-tau to predict AD in the prodromal stage of the disease, together with a high specificity to differentiate AD from stable MCI cases and those developing other dementias, such as frontotemporal dementia and Lewy body dementia (74). High performance of the core AD CSF biomarkers for prodromal AD was later verified in several large multi-centre studies such as the Descripa study (75), the ADNI study (76), and the Swedish Brain Power study (77).

Some studies have examined whether CSF biomarkers may aid in predicting AD pathology already in the preclinical stage of the disease. In 2003, a population-based study showed that CSF Aβ42 is lowered in cognitively unimpaired 85-year olds who later developed AD dementia, while there was no significant change in CSF Aβ40 (78), or CSF T-tau or P-tau levels. The finding that low CSF Aβ42, but not T-tau, predicts future cognitive decline and dementia in unimpaired elderly as long as 8 years in advance was verified in a subsequent paper (79). A clinical cohort study also showed that CSF Aβ42, but not T-tau and P-tau, predict future cognitive decline in the elderly (80). In a similar way, asymptomatic FAD mutation carriers FAD mutations have low CSF Aβ42 levels (81),

while one study also found the AD CSF profile of low CSF Aβ42 and high T-tau and P-tau in asymptomatic mutation carriers (82). These findings support that lowering of CSF Aβ42 is a very early indicator of clinically silent brain amyloidosis.

The core AD CSF biomarkers enter diagnostic criteria

Historically, AD is classified into the group of brain disorders called "dementias", which refers to cognitive symptoms severe enough to interfere with social or occupational activities. Following the dementia concept, clinical diagnostic criteria for AD were published by the Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) in 1984 (83). These were clinical exclusion criteria for AD in the dementia stage warranting a neuropathological examination after death to make a definite diagnosis. A diagnosis of "probable AD" could be set in patients aged 40-90 years, with progressive dementia after exclusion of other disorders than AD that could accounts for the deficits in memory and cognition, while a diagnosis of "definite AD" could only be set after death and autopsy investigation (83). It should be noted that an exclusion diagnosis was the only way possible, given that no fluid or imaging biomarkers were available at that time point. In the 1990s, the earlier clinical stages of AD gained increasing attention in clinical AD research, and the new term mild cognitive impairment (MCI) was introduced, referring to the transitional state between normal ageing and dementia (84). Similar to dementia, MCI is an etiologically heterogeneous syndrome that can be due to many underlying diseases, with only around half of cases having AD pathology (85).

An important first step towards a biological definition of AD came in 2007, when the International Working Group (IWG) led by Bruno Dubois published the first research criteria for the diagnosis of prodromal AD (86). First, these criteria provided the conceptual framework to allow a diagnosis of AD in patients with a very mild clinical phenotype of episodic memory disturbances, *i.e.*, before the dementia stage. Second, these criteria employed biomarkers (one or more of CSF Aβ42 and tau proteins, volumetric MRI and amyloid PET (86). Criteria based on similar principles for MCI due to AD (87) and dementia due to AD (88) were also published by the National Institute on Aging - Alzheimer's Association (NIA-AA) working groups on diagnostic guidelines for AD. Recently, an update of the IWG criteria was published (89), in which CSF biomarkers (low Aβ42 combined with high either T-tau or P-tau) together with amyloid PET had a more central role, while topographical biomarkers (volumetric MRI and FDG-PET) were assigned as tools to monitor neurodegeneration and the disease course in the disease. This year, the National Institute on Aging and Alzheimer's Association (NIA-AA) work group has now defined AD as a pathologic process that is identified primarily by biomarkers (REF – Jack et al). In this framework,

biomarkers are grouped into β-amyloid deposition, tau pathology, and neurodegeneration, following the A/T/N classification (90). In the new NIA-AA definition of AD, the clinical consequences of the disease (*i.e.*, cognitive symptoms) will only be used for staging purposes (REF – Jack et al).

Efforts to make the AD biomarker assays reach the Clinical Chemistry grade

Although numerous clinical studies reported excellent diagnostic performance of core AD CSF biomarkers measure by ELISA methods (1-3) or by the Luminex technology (91), it became evident that there was a marked difference in absolute levels reported between studies, also when using the same ELISA variant (92), with between-laboratory variability being more pronounced for CSF Aβ42 than for T-tau or P-tau (93). These types of differences in absolute levels may be caused by different pre-analytical procedures (*e.g.*, type of test tube for CSF collection or freeze-thaw schedule) across clinics and laboratories (94). In addition, between laboratory variability may also be due to discrepancies in analytical procedures between laboratories, or in the manufacturing procedures for the immunoassays, resulting in batch-to-batch variation.

The Alzheimer's Association quality control program

Strict quality control procedures in a single clinical laboratory can assure correct measurements over time (36) and be the basis for the implementation of CSF biomarker assays in clinical routine diagnostics (95). However, differences in absolute levels between laboratories precludes the introduction of uniform global cut-off levels and hinders a widespread implementation of CSF biomarkers in clinical routine. Therefore, the Alzheimer's Association quality control (QC) program for CSF biomarkers (93) was launched in 2009, with the aim to establish a platform to monitor the performance of the CSF biomarker measurements between laboratories and between batches of reagents. Between-laboratory and between-batch variability is not unique for the AD CSF biomarkers, and the design of the QC program is also similar to other proficiency programs for routine biomarker assays in Clinical Chemistry, with aliquots from the same pools being sent out to participants, and biomarker reported back, and summary forms presented by the organizers. Disappointingly, the between-laboratory CVs have consistently been 15-25% since start, despite standardization and training efforts, including introduction of standard operating procedures (SOPs) for the ELISA methods (96), denoting the need of more precise and automated analytical techniques.

Reference methods and materials

The highest level of standardization in Clinical Chemistry is through a Certified Reference Material (CRM), meaning (in our case) a "Gold Standard" CSF pool with certified biomarker levels, from which aliquots are distributed to biotech companies for harmonization of biomarker levels

between assay formats, and to assure low batch-to-batch variability for the assays. In 2009, the the International Federation of Clinical Chemistry and Laboratory Medicine Working Group for CSF proteins (IFCC WG-CSF), http://www.ifcc.org/ifcc-scientific-division/sd-working-groups/csf-proteins-wg-csf/, was formed (Figure 1). Knowing that Aβ42 was the most problematic AD CSF biomarker, the WG started working on standardization of this biomarker (97) in close conjunction with the Alzheimer's Association Global Biomarker Standardization Consortium (GBSC) (98).

To allow measurement of the absolute concentration of Aβ42 in CSF, an analytical method capable of absolute quantification without matrix effects was needed. Within the IFCC WG-CSF, two similar, but not identical, Reference Measurement Procedures (RMP) based on selected reaction monitoring (SRM) mass spectrometry, in which isotope-labelled Aβ42 spiked into to the CSF sample as an internal standard prior to sample workup, were developed (99-101). These candidate methods were then fully validated and received approval by the Joint Committee for Traceability in Laboratory Medicine (JCTLM) (102), which is the regulatory body for reference methods, as RMPs for CSF Aβ42 (No. C11RMP9 and C11RMP9).

For the development of the Aβ42 CRM, commutability studies showed that only native human CSF would work, since different variants of artificial CSFs with spiked Aβ42 did not behave like the native peptide when present in human CSF (103). In collaboration with the Joint Research Centre (JRC) European Commission Science Hub, see https://ec.europa.eu/jrc/en/reference-materials, the IFCC WG-CSF produced three CRMs with low, medium and high CSF Aβ42 levels, with certified concentrations assigned using the RMPs after evaluation of concordance in Round Robin studies (49). These three CRMs (meaning large sets of CSF aliquots) have also been evaluated for quality measures such as homogeneity and long-term stability. The CRMs will serve at the top of the calibration hierarchy, with the aim to calibrate commercial assays to these reference aliquots, and make absolute levels obtained using different assays comparable.

CSF biomarkers on fully automated instruments

As reviewed above, the between-laboratory variability for the AD CSF biomarkers seen in the Alzheimer's Association QC program showed the need to develop assays for these proteins on fully automated laboratory instruments, just like for many protein biomarkers (e.g. troponin-T for myocardial infarction and TSH for thyroid disorders). These instruments involve no manual steps, and assays generally have superior performance as compared with ELISA methods.

In 2016, the first paper on the full validation and analytical performance the Cobas Elecsys β-amyloid (1-42) assay was published (104). This assay showed excellent performance and very low batch-to-batch variability, and has also been running in the QC program since 2014, with between laboratory coefficients of variations (CVs) of a few percent as compared with 15-20% for the ELISA methods. In addition, novel assays for T-tau and P-tau on the Elecsys instrument was also recently published (37), and have done two rounds in the QC program, showing excellent CVs of 2.7% (T-tau) and 1.8% (P-tau). Other companies, such as Fujirebio and Euroimmun, have followed and built assays on fully automated laboratory platforms, also showing highly improved performance in the QC program.

Future developments likely include that fully automated laboratory analyzers assays will prove to give stable and precise results for the AD CSF biomarkers between laboratories, which, together with CRMs will allow for the establishment of uniform worldwide cut-off levels. This will be important both in the routine diagnostic evaluation of patients with suspected AD, and in the diagnostic procedure for inclusion in clinical trials in novel disease-modifying drugs. Further, stable and exact CSF biomarker levels will allow for merging data from clinical research studies world-wide, in clinical studies on disease pathogenesis.

Synaptic biomarkers for AD

Although the core CSF AD biomarkers reflect central pathogenic mechanisms of the disease, novel biomarkers to monitor additional important molecular mechanisms in AD are constantly sought. One important component of AD pathologic change and pathophysiology is synaptic dysfunction and degeneration. Synapses are the central communication units in the neuronal networks the brain. Synapses consist of a pre-synaptic domain, where synaptic vesicles that contain the neurotransmitters that are released upon activation are located. Neurotransmitter release is a process regulated by a delicate machinery of specific pre-synaptic proteins (105). After release to the synaptic cleft, neurotransmitters bind to post-synaptic receptors at the dendritic spines and activate a cascade of molecular events to advance the neuronal signal (106). Synaptic dysfunction and degeneration are likely the direct cause of the cognitive deterioration in AD.

A large body of literature supports a marked degeneration and loss of synapses in grey matter regions in AD, also in the early disease stages (107, 108). Importantly, severity of synaptic loss is more tightly correlated with degree of cognitive impairment than either plaque or tangle counts (109-111), and synaptic degeneration has been suggested as the best anatomical correlate of cognitive deficits in AD (109, 112). Further, experimental animal studies suggest that both Aβ

fibrils (113) and diffusible Aβ oligomers (114) may disturb dendritic spines by distinct mechanisms. In addition, tau hyperphosphorylation and microglia activation may also contribute to spine loss (115, 116). Thus, synaptic biomarkers in CSF may serve as tools to explore this important aspect of AD pathophysiology in man, and to examine the link between effects on AD molecular pathology and cognitive symptoms by novel drug candidates with disease-modifying potential. Synapses are plastic structures in the brain and, potentially, synaptic markers would change rapidly in response to successful treatment.

Early search for synaptic proteins in CSF

Based on semi-preparative scale chromatographic and gel electrophoretic protein separation combined with Western blotting and mass spectrometric identification, we were in the late 1990ies able to identify synaptic proteins in CSF from the key synaptic compartments, including the presynaptic vesicle proteins synaptotagmin and rab3a, the presynaptic membrane protein SNAP-25, and the dendritic protein neurogranin (117, 118). These discoveries served as the motivation to initiate a project on production on novel antibodies and detailed mass spectrometric characterization of synaptic proteins in human CSF aiming at developing quantitative immunoassays for reliable quantification in individual samples. A first pilot study in 2010, based on semi-quantitative immunoprecipitation combined with Western blotting showed promising results with a marked increase in CSF neurogranin in AD (119).

Dendritic proteins - neurogranin

Dendritic spines are specialized protrusions on the dendrites, the point where neurons receive and integrate information. Neurogranin is a dendritic protein, expressed in the cortex and hippocampus by excitatory neurons (120, 121), and is known to play an important role in long-term potentiation (122, 123). Neurogranin expression is highest in associative cortical areas, but levels are markedly reduced in the hippocampus and the frontal cortex in AD, indicating loss of post-synaptic elements (124, 125). Thus, measurement of neurogranin in CSF may serve as a biomarker for dendritic instability and synaptic degeneration.

After developing novel monoclonal antibodies to measure neurogranin by ELISA, high CSF levels were found to predict prodromal AD in MCI (126). High CSF neurogranin in AD dementia and prodromal AD has been confirmed in several subsequent papers (127, 128), including in the ADNI study (129). High CSF neurogranin also correlates with future rate of hippocampal trophy measured by MRI and rate of metabolic reductions on FDG-PET (129). Interestingly, a recent study suggests that high CSF neurogranin may be specific for AD, and not found in other

neurodegenerative disorders such as frontotemporal dementia, Lewy body dementia, Parkinson disease, progressive supranuclear palsy, or multiple system atrophy (130).

Mass spectrometry characterization of neurogranin in CSF suggests that it is present in CSF as a series of C-terminal peptides (126), while other studies using a sandwich immunoassay combining N- and C-terminal antibodies, which thus measures full-length neurogranin (131), as well as an assay specific for neurogranin peptides ending at position 75 (132), also found high CSF levels in AD and MCI as compared with controls Thus, we need further studies on how neurogranin is processed and released from neurons into the CSF, including studies comparing the diagnostic potential of full-length versus C-terminal neurogranin peptides.

Presynaptic biomarkers

In the presynaptic terminal, the SNARE complex proteins, including synaptosomal-associated protein 25 (SNAP-25), syntaxin-1, and vesicle-associated membrane protein (VAMP)/synaptobrevin, are key components of the molecular machinery that drives fusion of membranes in neurotransmitter exocytosis (133). While SNAP-25 is located at the synaptic vesicles, synaptotagmin-1 (SYT1) is found in the presynaptic plasma membrane, and is essential for synaptic vesicle exocytosis, and thus neurotransmitter release (134).

The levels of both SNAP-25 and SYT1 are reduced in cortical areas in the AD brain (124, 135), reflecting the synaptic degeneration and loss in AD. Interestingly, using immunoprecipitation mass spectrometry methods, a marked increase in the CSF levels of both SNAP-25 and SYT1 was found in AD dementia and prodromal AD cases (135, 136). These promising results need validation in future studies, but suggest that a set of synaptic proteins covering different components of the synaptic unit (dendrites – neurogranin, presynaptic plasma membrane – SNAP-25, synaptic vesicles – SYT1) may be valuable tools in clinical studies on the relevance of synaptic dysfunction and degeneration in AD pathogenesis, and maybe also in the clinical evaluation of patients.

Blood biomarkers for AD

Since blood is more accessible than CSF, there is little doubt that blood sampling would be preferable to CSF when it comes to taking fluid samples to measure AD biomarkers, both for clinical diagnosis or screening and for repeated sampling in clinical trials. However, developing blood biomarkers for AD has proven difficult; while the CSF is continuous with the brain extracellular fluid, with a free exchange of molecules from the brain to the CSF, only a fraction of brain proteins enters the bloodstream. Further, blood is a more challenging matrix than CSF for

brain biomarkers, for several reasons. First, the minute amounts of brain proteins entering the blood have to be measured in a matrix containing very high levels of plasma proteins, such as albumin and IgG, introducing a high risk of interference in analytical methods (137). Second, in addition to dilution, brain proteins released into blood may be degraded by proteases, metabolized in the liver or cleared by the kidneys, which will introduce a variance that is unrelated to brain changes and difficult to control for. This limits the potential of finding blood biomarkers for AD (138). Nevertheless, technical developments in the field of ultrasensitive immunoassays and mass spectrometry have given new hopes (139).

Aβ in plasma

While numerous papers on CSF Aβ42 consistently have found a high concordance with amyloid PET measures of plaque burden (35), and a marked decrease in AD, studies on plasma Aβ42 as a biomarker reflecting brain amyloid pathology (and thus AD) have been disappointing, with contradictory results, with no or minor changes and large overlaps in both Aβ42 and Aβ40 levels between patients and controls (30). This lack of association with disease pathology may be due to the contribution from peripheral tissues to plasma Aβ, as also evidenced by the lack of correlation between plasma and CSF Aβ concentrations (140). The poor disease association might also be related to analytical shortcomings using ELISA methods or other standard immunoassays, *e.g.*, epitope masking by hydrophobic Aβ peptides binding to plasma proteins (141), or other interferences that might be mitigated by analytical improvements.

In 2011, we published a novel method based on the Single molecule array (Simoa) technique for measurement of Aβ42 in plasma (142). This technique is based on immunocapture of the protein biomarker on magnetic beads, which are trapped in femto-liter volume wells, followed by addition of enzyme-labelled detection antibody and digital quantification that allows for exact quantification of Aβ42 down to sub-picogram per mL levels (limit of quantification of 0.04 pg/mL). The high analytical sensitivity allows for pre-dilution of samples that may reduce matrix interferences. When evaluating this assay in the large Swedish BioFINDER study cohort, weak but significant correlations were found between both plasma Aβ42 and the Aβ42/40 ratio and the corresponding CSF measures, as well as to cortical $^{[18F]}$flutemetamol PET retention (143). Significantly lower plasma Aβ42/40 ratio ($p<0.002$) was found in both MCI and AD cases as compared with controls.

In an attempt to evaluate if mass spectrometric analysis may give a more accurate quantification of Aβ peptides in plasma, we developed an immunoprecipitation (IP) mass spectrometry (MS) selected reaction monitoring (SRM) method for quantification of Aβ42 and Aβ40, where stable

isotope-labelled Aβ peptides are added to the sample before analysis (and thus processed and analysed simultaneously with endogenous Aβ peptides) and using the detergent octyl-glucopyranoside to disrupt complexes between Aβ and plasma proteins such as albumin (144). In a small pilot clinical study based on clinically diagnosed cases, we were not able to find any significant change, even if there was an apparent trend for a reduction on both plasma Aβ42 and the Aβ42/40 ratio in AD (144). Interestingly, using a similar IP-MS method, also involving LysN proteolytic digestion of Aβ peptides before analysis, significantly lower Aβ42 concentration and Aβ42/40 ratio was found in amyloid PET-positive compared with -negative cases (145). The Aβ42/40 ratio was 14% lower in the amyloid PET positive group, which gave an impressive ROC value of 0.89 (145). Additional MS-based studies suggest that a ratio of a certain APP fragment (APP669-711) to Aβ42 or Aβ42/Aβ40 in plasma identifies Aβ-positive individuals with high sensitivity and specificity (refs: Kaneko, N., et al., Novel plasma biomarker surrogating cerebral amyloid deposition. Proc Jpn Acad Ser B Phys Biol Sci, 2014. 90(9): p. 353-64; Nakamura, A., et al., High performance plasma amyloid-beta biomarkers for Alzheimer's disease. Nature, 2018. 554(7691): p. 249-254). Nakamura and colleagues have described the sensitivity and specificity of predicting 11C-PiB in AD and MCI patients, as well as in cognitively normal individuals, at 91% and 87%, respectively (ref: Nakamura, A., et al., High performance plasma amyloid-beta biomarkers for Alzheimer's disease. Nature, 2018. 554(7691): p. 249-254). These promising results call for further studies to evaluate plasma Aβ as a screening tool for brain amyloidosis and AD, also including larger clinical cohorts and comparisons of different analytical platforms for measurement.

Tau protein in plasma

Ultrasensitive immunoassay techniques also allow for measurement of tau protein in blood samples (139), with increased tau levels in plasma in AD found using both the immuno-magnetic reduction (IMR) (146) and Simoa (147), methods. A large study on both the ADNI and BIOFINDER cohorts could confirm an increase in plasma tau concentrations in AD dementia, although with a substantial overlap in levels with controls (148). Interestingly, longitudinal data showed significant correlations between plasma tau levels and future cognitive decline, as well as increases in atrophy measured by MRI and in hypometabolism measured by FDG PET during follow-up (148). Thus, current data suggest a minor increase in plasma tau in AD, although with too large overlap with controls to be diagnostically useful. Tau protein in CSF has been found to be present as truncated fragments (149), and it is possible that development of assays based on antibodies for specific tau fragments will improve performance. Alternatively, measurement of T-tau or P-tau in neuron-enriched exosome preparations may improve performance for tau as a blood biomarker (150), but further studies are needed to validate this finding.

Neurofilament light in plasma

We have also developed a highly sensitive Simoa method for the axonal protein neurofilament light (NFL) protein (151). This assay has many-fold higher analytical sensitivity than assays using the same anti-NFL antibodies based on the electrochemiluminescence (ECL) Meso Scale Diagnostics (MSD) technique or standard ELISA (152), meaning that NFL can be measured also in blood samples from normal individuals who have plasma NFL concentrations that are below the level for accurate quantification when using ECL-MSD or ELISA. In contrast to tau protein, the correlation between plasma and CSF levels of NFL protein is tight (151).

A recent study on the ADNI cohort showed a marked increase in plasma NFL in AD cases (149% of control levels), with a receiver operating characteristic (ROC) area under curve (AUC) value of 0.87, which is comparable to the core AD CSF biomarkers (153). While the change in the MCI group was less pronounced, plasma NFL was highest MCI cases with positive amyloid PET scans, and predicted faster cognitive deterioration, higher rate of future both brain atrophy (measured by MRI) and hypometabolism as measured by FDG-PET (153). Importantly, in a study on 48 familial AD (FAD) mutation carriers and non-carriers, blood NFL was increased in symptomatic FAD cases, but also in pre-symptomatic mutation carriers, with levels correlating with expected estimated year of symptom onset as well as both cognitive and MRI measures of disease stage (Weston 2017). These results indicate that blood NFL detects neurodegeneration also in the preclinical stage of AD.

In this context, an important piece of knowledge is that high plasma (or CSF) NFL is not a feature that is specific for AD. Instead, increased levels are found in many neurodegenerative disorders, such as frontotemporal dementia, progressive supranuclear palsy and corticobasal syndrome (154, 155). Thus, a possible future application for plasma NFL is as a screening test at the first clinical evaluation of patients with cognitive disturbances, e.g. at the primary care unit. Here, plasma NFL might serve as simple, non-invasive, and cheap screening tool, primarily to rule out neurodegeneration.

Concluding remarks

The last 20 years have seen an enormous expansion in research on fluid biomarkers for AD. The core AD CSF biomarkers T-tau, P-tau and Aβ42 (and the Aβ42/40 ratio) have been evaluated in hundreds of clinical neurochemical studies with extraordinary consistent results, showing high diagnostic accuracy both for AD dementia, but importantly also for prodromal AD. These biomarkers have undergone a phase of standardization and new assay versions on fully

automated instruments show excellent analytical performance and low intra- and inter-laboratory variation. The core AD biomarkers are today part of research diagnostic criteria, and we foresee an increased use of these diagnostic tests in clinical routine practice. The AD CSF biomarker toolbox has been expanded with novel biomarkers reflecting additional aspects of AD pathology, such as synaptic dysfunction.

We envision further development and validation of assays reflecting other pathologies common in age-related neurodegenerative disorders, including Lewy body and TDP-43 pathology, reaching the stage of clinical applications in the coming years, so that CSF biomarkers can be part in a personalized medicine approach to improve the evaluation of patients with cognitive disturbances. Last, we hope that blood biomarkers may be implemented as screening tools in the first-in-line clinical evaluation of this group of patients, once we have disease-modifying drugs at hand.

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